REMARKS

Rejections under 35 U.S.C. §101

All claims have been rejected under 35 U.S.C. § 101 as reading on nonstatutory subject matter. Applicants have limited the claims by amendment herein to methods of making transgenic **non-human** mammals, as per the Examiner's suggestion, and respectfully submit that the rejection has been obviated by amendment and should be withdrawn.

Rejections under 35 U.S.C. §112, ¶ 1, Scope of Enablement

The Examiner rejects all pending claims under 35 U.S.C. § 112, first paragraph, in three separate scope of enablement rejections. Applicants respectfully traverse.

Scope of "phenotype"

Noting that "[t]he claimed invention is drawn to methods of making transgenic mammals with no particular phenotype," and that applicants' specification similarly "discloses no phenotype for the genetically modified mammals," the Examiner concludes that "undue experimentation would have been required for one of skill in the art to make and use the claimed invention."

The Examiner's observations are correct: applicants' claimed methods are generic to, and thus read on, any user-desired phenotype, and the methods taught in applicants' specification are of commensurate scope.

The conclusion that the Examiner draws from these observations, however, is incorrect, and manifestly so.

Were it otherwise, were it always necessary to claim methods of transgenesis in association with a "particular phenotype", the following claims would perforce equally be said to be invalid under 35 U.S.C. § 112, first paragraph:

A method of producing a transgenic bovine, the method comprising:

obtaining an ovum from bovine ovaries;

maturing the ovum in vitro;

fertilizing the mature ovum in vitro to form a zygote;

introducing a transgene into the zygote in vitro;

maturing the zygote to a preimplantation stage embryo in vitro; and

transplanting the embryo into a recipient female bovine, wherein the female bovine gestates the embryo to produce a transgenic bovine.[1]

A method for regenerating a fertile transformed wheat plant to contain foreign DNA comprising the steps of:

(a) producing regenerable tissue from said plant to be transformed:

(b) transforming said regenerable tissue with said foreign DNA where said foreign DNA comprises a selectable DNA sequence, where said sequence functions in a regenerable tissue as a selection device;

(c) between about one day to about 21 days after step b) placing said regenerable tissue from step b) in a medium capable of producing shoots from said tissue wherein said medium further contains a compound used to select regenerable tissue containing said selectable DNA sequence to allow identification or selection of the transformed regenerated tissue;

(e) after at least one shoot has formed from the selected tissue of step c), transferring said shoot to a second medium capable of producing roots from said shoot to produce a plantlet; and,

(e) growing said plantlet into a fertile, transgenic wheat plant wherein the foreign DNA is transmitted to progeny plants in mendelian fashion.[2]

A method for producing a transgenic pig comprising:

a) obtaining a porcine embryo comprising at least three blastomeres;

b) introducing at least one clone of isolated nucleic acid molecules into at least one blastomere of the embryo;

c) transferring the embryo to a surrogate female pig;

d) developing the embryo into at least the fetal stage; and

e) developing the fetus into a transgenic pig.[3]

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[1] Claim 1, U.S. Pat. No. 5,633,076.

[2] Claim 1, U.S. Pat. No. 5,631,152.

A method of genetically engineering a conifer, the method comprising the steps of

placing immature embryos of the conifer on a target surface;

physically accelerating at the embryos carrier particles which are much smaller than the cells of the embryos, the carrier particles carrying copies of a foreign genetic construction including at least one foreign gene of interest and one selectable marker gene;

inducing the embryos to form proliferative callus which is capable of forming somatic embryos;

during the step of inducing, culturing the callus in a medium containing a selection agent for which the selectable marker gene confers resistance so as to select for embryogenic callus which is totally transformed and which expresses the gene of interest;

inducing somatic embryos to form from the callus; and

regenerating the somatic embryos thus produced into clonal transgenic conifer plants.[4]

A method of producing a transgenic mouse having a genome comprising a modification of a target DNA sequence, said method comprising:

(a) transforming a population of mouse embryonic stem cells with a PNS vector;

(b) identifying a cell having said genome by selecting for cells containing said positive selection marker and against cells containing said negative selection marker and analyzing DNA from cells surviving selection for the presence of said modification;

(c) inserting said cell into a mouse embryo;

(d) propagating a mouse from said embryo; and

(e) breeding said mouse to produce said transgenic mouse having said genome;

wherein said PNS vector comprises:

(1) a first homologous vector DNA sequence capable of homologous recombination with a first region of said target DNA sequence,

(2) a positive selection marker DNA sequence capable of conferring a positive selection characteristic in said cells,

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[3] Claim 1, U.S. Pat. No. 6,498,285.

[4] Claim 1, U.S. Pat. No. 5,681,730.

(3) a second homologous vector DNA sequence capable of homologous recombination with a second region of said target DNA sequence, and

(4) a negative selection marker DNA sequence, capable of conferring a negative selection characteristic in said cells, but substantially incapable of homologous recombination with said target DNA sequence,

wherein the spatial order of said sequences in said PNS vector is: said first homologous vector DNA sequence, said positive selection marker DNA sequence, said second homologous vector DNA sequence and said negative selection marker DNA sequence as shown in FIG. 1,

wherein the 5'-3' orientation of said first homologous vector sequence relative to said second homologous vector sequence is the same as the 5'-3' orientation of said first region relative to said second region of said target sequence;

wherein the vector is capable of modifying said target DNA sequence by homologous recombination of said first homologous vector DNA sequence with said first region of said target sequence and of said second homologous vector DNA sequence with said second region of said target sequence.[5]

A method of transforming a corn plant cell or plant tissue using an Agrobacterium mediated process comprising the steps of:

inoculating a transformable plant cell or tissue from a corn plant with Agrobacterium containing at least one genetic component capable of being transferred to the plant cell or tissue in an inoculation media containing an effective amount of at least one antibiotic that inhibits or suppresses the growth of Agrobacterium;

co-culturing the transformable plant cell or tissue after the inoculating step in a medium capable of supporting growth of plant cells or tissue expressing the genetic component, said medium not containing said antibiotic;

selecting transformed plant cells or tissue; and

regenerating a transformed corn plant expressing the genetic component from the selected transformed plant cells or tissue.[6]

A method for the production of fertile, transgenic plants wherein the transgenic plant has a DNA sequence of interest integrated at a

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[5] Claim 1, U.S. Pat. No. 5,627,059.

[6] Claim 1, U.S. Pat. No. 6,603,061.

predetermined DNA sequence of the plant, said method comprising the steps of

introducing into plant cells a DNA construct comprising

a multifunctional DNA sequence flanked by a pair of directly repeated site-specific recombination sequences,

said multifunctional DNA sequence comprising a gene encoding a selectable marker, and a DNA sequence of interest,

wherein said DNA sequence of interest is flanked by nucleotide sequences sharing homology to the predetermined nucleotide sequence present in the plant cell, and the selectable marker gene is operably linked to regulatory sequences capable of expressing the gene in the plant cell,

selecting for plant cells having said DNA construct integrated into the DNA of the plant cell,

eliminating randomly inserted DNA constructs through expression of a recombinase gene capable of initiating recombination at the site-specific recombinase sequences in the plant cells,

identifying cells having said DNA sequence of interest integrated into the plant's DNA via a homologous recombination event, and

culturing said identified cells to generate an entire plant.[7]

A method for producing transgenic wheat plants, comprising the steps:

(a) obtaining a Type C embryogenic wheat callus;

(b) delivering heterologous DNA into the cells of said callus by bombarding said callus with accelerated microprojectiles adsorbed with said DNA;

(c) selecting for and growing transgenic wheat cells; and

(d) regenerating transgenic wheat plants from said transgenic wheat cells.[8]

A method of making a non-human mammal harboring a biologically functional non-native cell comprising:

(i) selectively destroying the endogenous germ cell population in the seminiferous tubules of a first non-human mammal, leaving intact the supporting cells comprising Sertoli cells in said tubules; and

(ii) colonizing said seminiferous tubules of said first non-human mammal with spermatogonia from a second non-human mammal of the same species as the first non-human mammal, wherein said colonizing

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[7] Claim 1, U.S. Pat. No. 5,527,695.

[8] Claim 1, U.S. Pat. No. 5,405,765.

comprises injecting a solution containing said spermatogonia from said second mammal into said seminiferous tubules or into the lumen of the rete testes into the efferent duct leading into the epididymis of said first mammal,

wherein following colonization, said spermatogonia from second mammal produce spermatozoa which are capable of repopulating said seminiferous tubules, fertilizing ova, and producing viable offspring.[9]

Were it always necessary to claim methods of transgenesis in association with a "particular phenotype", all of those claims would have to be said to be invalid under 35 U.S.C. § 112, first paragraph. And they cannot: each is statutorily presumed to be enabled across the entirety of its scope, 35 U.S.C. § 282; 35 U.S.C. § 112, ¶ 1, and the Examiner is prohibited from adducing any argument that would cast the validity of such claims in doubt, M.P.E.P. § 1701.

And that is of course the correct result: it should be no more necessary to limit claims drawn to improved methods of transgenesis to a particular transgenic phenotype than it is necessary to limit claims drawn to improved methods of nucleic acid sequencing to a particular nucleic acid to be sequenced, or to limit claims drawn to improved methods of propagating hybridomas to a particular specificity of secreted antibody, or to limit claims drawn to improved methods of weighing solids to a particular type of solid to be weighed. Indeed, nothing in the first paragraph of section 112 mandates that the genotypic alteration effected by applicants' claimed methods change the transgenic animal's immediately observable phenotype *at all.*[10]

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[9] Claim 1, U.S. Pat. No. 5,858,354.

[10] The Examiner's echo of *Brenner v. Manson*, that "a method of making a product has use only if the product made has use," is best answered thus: the methods here claimed, and the products thereof, have all of the uses, respectively, as do the transgenesis methods of earlier-issued claims that are similarly broad to phenotype.

Imagine claim 32 recast in Jepson format: "A method of producing transgenic non-human mammals, the improvement comprising: introducing into at least one mammalian zygote at least

The question is not whether applicants are permitted to claim improved methods that are broad to any desired phenotype -- indeed, sufficiently broad to read on a transgenic animal with a wild-type phenotype -- but, rather, whether applicants' specification, coupled with information known in the art, is sufficient to enable applicants' methods across a sufficient number of genetic (not *phenotypic*) alterations to meet the requirements of 35 U.S.C. § 112, first paragraph.

Applicants respectfully submit that it does, and that the Examiner has adduced neither evidence nor reasoned argument to suggest otherwise. With respect, the Examiner has failed to establish a *prima facie* case of inadequate scope of enablement, the rejection is in error, and the rejection should be withdrawn.

Scope of "genotype"

The Examiner rejects claims 32 - 37 and 41 - 70 on grounds that, "[w]hile the specification enables single base substitutions, no guidance is provided for making other types of changes to the genome" such as "inserting entire genes into specifically selected sites of the target genome."

Applicants respectfully disagree: the specification clearly discloses that the methods of the present invention may be used, *inter alia*, to effect just such insertions. By way of nonlimiting example,

> In general, transgenic animals are made with any number of changes. Exogenous sequences, or extra copies of endogenous sequences, including structural genes and regulatory sequences, may be added to the animal, as outlined below. Endogenous sequences (again, either genes or regulatory sequences) may be disrupted, i.e. via insertion, deletion or substitution, to prevent expression of endogenous proteins. Alternatively, endogenous sequences may be modified to alter their biological function,

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one recombinase and at least two single-stranded targeting polynucleotides . . . . " Unless the introduction of recombinase in concert with the targeting polynucleotides is less efficacious than introduction of the transgene alone, the method of claim 32 can be no less useful than a method, lacking use of a recombinase, that is earlier-claimed.

for example via mutation of the endogenous sequence by insertion, deletion or substitution.

Accordingly, [t]he methods of the present invention are useful to add exogenous DNA sequences, such as exogenous genes or regulatory sequences, extra copies of endogenous genes or regulatory sequences, or exogenous genes or regulatory sequences, to a transgenic plant or animal. This may be done for a number of reasons: for example, adding one or more copies of a wild-type gene can increase the production of a desirable gene product; adding or deleting one or more copies of a therapeutic gene can alleviate a disease state, or to create an animal model of disease. Adding one or more copies of a modified wild type gene may be done for the same reasons. Adding therapeutic genes or proteins may yield superior transgenic animals, for example for the production of therapeutic or nutriceutical proteins. Adding human genes to non-human mammals may facilitate production of human proteins and adding regulatory sequences derived from human or non-human mammals may be useful to increase or decrease the expression of endogenous or exogenous genes. Such inserted genes may be under the control of endogenous or exogenous regulatory sequences, as described herein.[11]

In a preferred embodiment, an insertion sequence comprises a gene which not only disrupts the endogenous gene, thus preventing its expression, but also can result in the expression of a new gene product. Thus, in a preferred embodiment, the disruption of an endogenous gene by an insertion sequence gene is done in such a manner to allow the transcription and translation of the insertion gene. An insertion sequence that encodes a gene may range from about 50 bp to 5000 bp of cDNA or about 5000 bp to 50000 bp of genomic DNA. As will be appreciated by those in the art, this can be done in a variety of ways. In a preferred embodiment, the insertion gene is targeted to the endogenous gene in such a manner as to utilize endogenous regulatory sequences, including promoters, enhancers or a regulatory sequence. In an alternate embodiment, the insertion sequence gene includes its own regulatory sequences, such as a promoter, enhancer or other regulatory sequence etc.

Particularly preferred insertion sequence genes include, but are not limited to, genes which encode therapeutic and nutriceutical proteins, and reporter genes. Suitable insertion sequence genes which may be inserted into endogenous genes include, but are not limited to, nucleic acids which encode those genes listed as suitable endogenous genes for alterations, above, particularly mammalian enzymes, mammalian antibodies, mammalian proteins including serum albumin as well as mammalian therapeutic genes. In a preferred embodiment, the inserted mammalian

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[11] Specification, p. 45, lines 8 - 29.

gene is a human gene. Suitable reporter genes are those genes which encode detectable proteins, such as the genes encoding luciferase, .beta.-galactosidase (both of which require the addition of reporter substrates), and the fluorescent proteins, including green fluorescent protein (GFP), blue fluorescent protein (BFP), yellow fluorescent protein (YFP), and red fluorescent protein (RFP).

Thus, in a preferred embodiment, the targeted sequence modification creates a sequence that has a biological activity or encodes a polypeptide having a biological activity. In a preferred embodiment, the polypeptide is an enzyme with enzymatic activity. In another preferred embodiment, the polypeptide is an antibody. In a third preferred embodiment, the polypeptide is a structural protein.

In addition, the insertion sequence genes may be modified or variant genes, i.e. they contain a mutation from the wild-type sequence. Thus, for example, modified genes including, but not limited to, improved therapeutic genes, modified (.alpha.-lactalbumin genes that do not encode any phenylalanine residues, or human enzyme or human antibody genes that do not encode any phenylalanine residues.[12]

Typically, a targeting polynucleotide (or complementary polynucleotide pair) has a portion or region having a sequence that is not present in the preselected endogenous targeted sequence(s) (i.e., a nonhomologous portion or mismatch) which may be as small as a single mismatched nucleotide, several mismatches, or may span up to about several kilobases or more of nonhomologous sequence. Generally, such nonhomologous portions are flanked on each side by homology clamps, although a single flanking homology clamp may be used. Nonhomologous portions are used to make insertions, deletions, and/or replacements in a predetermined endogenous targeted DNA sequence, and/or to make single or multiple nucleotide substitutions in a predetermined endogenous target DNA sequence so that the resultant recombined sequence (i.e., a targeted recombinant endogenous sequence) incorporates some or all of the sequence information of the nonhomologous portion of the targeting polynucleotide(s). Thus, the nonhomologous regions are used to make variant sequences, i.e. targeted sequence modifications. Additions and deletions may be as small as 1 nucleotide or may range up to about 2 to 4 kilobases or more. In this way, site directed modifications may be done in a variety of systems for a variety of purposes.[13]

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[12] Specification, p. 47, line 9 - p. 48, line 12.

[13] Specification, p. 53, lines 15 - 30.

It is beyond debate that applicants' specification clearly and unambiguously teaches that the methods of the present invention may be used to effect large insertions.

"[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." *In re Marzocchi*, 439 F.2d 220, 223 (C.C.P.A. 1971) (emphasis added); quoted with approval in *In re Brana*, 51 F.3d 1560 (Fed. Cir. 1995) .

To shoulder that burden, the Examiner notes comments in applicants' ***own specification*** that "as the region of heterology increases, the stability of four-strand hybrids decreases significantly," and concludes from that observation that "the efficiency of recombination would be expected to be much lower for larger deletions or insertions." Office Action at 3.

Logically, applicants' specification, read as a whole, must be understood to assert that, *notwithstanding* any decrease in stability of four-strand hybrids incident to increases in heterology, large insertions are nonetheless possible. The Examiner has adduced neither reason nor evidence sufficient to cast doubt on the objective truth of such statement.

Solely to expedite prosecution, admitting neither to the sufficiency of the Examiner's *prima facie* case of inadequate scope of enablement nor to an attendant shift of the burden of production to applicants, applicants respectfully invite the Examiner's attention to Maga *et al.*, "Increased efficiency of transgenic livestock production," *Transgenic Research* 12:485-496 (2003), attached hereto as Exhibit A. As reported, the use of RecA protein results in significant increase in transgene integration frequencies. The claimed methods work. They are fully enabled. The rejection is in error and should be withdrawn.

Scope of mammalian species

The Examiner rejects all pending claims under 35 U.S.C. § 112, first paragraph, on the ground that the specification fails to provide an enabling disclosure for making *targeted* changes to any mammal other than the mouse.

The USPTO has already established as fully enabled applicants' methods for targeting and altering, by homologous recombination, a pre-selected target DNA sequence in ***any eukaryotic cell*** to make a targeted sequence modification, the method comprising introducing into at least one eukaryotic cell at least one recA recombinase and at least two single-stranded targeting polynucleotides which are substantially complementary to each other, and which further comprise a homology clamp that substantially corresponds to or is substantially complementary to a preselected target DNA sequence. Claim 1, U.S. Pat. No. 5,763,240 (to which the instant application claims priority); 35 U.S.C. § 282; 35 U.S.C. § 112, first paragraph.

The rejection is in error and should be withdrawn.

Rejections under 35 U.S.C. § 112, ¶ 2

Claims 32 - 37 and 41 - 70 are rejected as indefinite under 35 U.S.C. § 112, ¶ 2, on grounds that the method steps recited in the body of the claims do not result in the transgenic mammal of the preamble. The rejection has been obviated by amendment of claim 32. No new matter has been added; support for the term "fostering" can be found in the specification at page 95, line 12.

Claims 32 - 37 and 41 - 70 are rejected as indefinite for recitation of modified endogenous nucleic acid", "because it is unclear relative to what standard or point of reference the endogenous nucleic acid is considered to be 'modified'", and because "it is unclear what would be regarded as an 'endogenous nucleic acid.'"

Applicants traverse the rejection: the terms are standard in the art and would be clearly understood by one skilled in that art.[14] Applicants respectfully request reconsideration.

Claims 34 and 65 are rejected as indefinite for recitation of "farm" mammal. The rejection has been obviated: claim 34 has been amended to recite the Markush group of livestock previously presented in claim 35, with cancellation of claim 35 and consequential amendment of claim 65.

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[14] Note, for example, claim 1 of U.S. Pat. No. 6,673,986:

1. A transgenic mouse comprising in its germline a modified genome wherein said modification comprises inactivated endogenous immunoglobulin heavy chain loci in which all of the J segment genes from both copies of the immunoglobulin heavy chain locus are deleted to prevent rearrangement and to prevent formation of a transcript of a rearranged locus and the expression of an endogenous immunoglobulin heavy chain from the inactivated loci.

CONCLUSION

Applicants submit that the present application is in condition for allowance, and respectfully request the same.

Respectfully submitted,

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Attachment (Exhibit A): Maga et al., "Increased efficiency of transgenic livestock production," *Transgenic Research* 12:485-496 (2003),

Transgenic Research 12: 485–496, 2003.
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EXHIBIT A

Increased efficiency of transgenic livestock production

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Abstract

Production of transgenic livestock by pronuclear microinjection of DNA into fertilized zygotes suffers from the compounded inefficiencies of low embryo survival and low integration frequencies of the injected DNA into the genome. These inefficiencies are one of the major obstacles to the large-scale use of pronuclear microinjection techniques in livestock. We investigated exploiting the properties of recombinase proteins that allow them to bind DNA to generate transgenic animals via pronuclear microinjection. In theory, the use of recombinase proteins has the potential to generate transgenic animals with targeted changes, but in practice we found that the use of RecA recombinase-coated DNA increases the efficiency of transgenic livestock production. The use of RecA protein resulted in a significant increase in both embryo survival rates and transgene integration frequencies. Embryo survival rates were doubled in goats, and transgene integration was 11-fold higher in goats and three-fold higher in pigs when RecA protein-coated DNA was used compared with conventional DNA constructs without RecA protein coating. However, a large number of the transgenic founders generated with RecA protein-coated DNA were mosaic. The RecA protein coating of DNA is straightforward and can be applied to any species and any existing microinjection apparatus. These findings represent significant improvements on standard pronuclear microinjection methods by enabling the more efficient production of transgenic livestock.

Introduction

The generation of transgenic animals, particularly mice, has become standard practice in many academic and commercial laboratories. Pronuclear microinjection of DNA into fertilized zygotes was the original technique used more than 20 years ago to generate the first transgenic animals (Gordon et al., 1980) and remains today as the main and simplest method of choice available for the production of many transgenic organisms. Pronuclear microinjection of DNA encoding a transgene has consistently been used to produce transgenic mice, rabbits, pigs, goats, and cows (Ebert & Schindler, 1993; Pursel & Rexroad, 1993). However, the production of transgenic livestock, in particular, is a very inefficient process and since its first use, few improvements have been made to increase the overall efficiency and effectiveness of pronuclear microinjection.

Transgenic animals produced by pronuclear microinjection typically contain multiple copies of the desired transgene integrated in tandem arrays at random sites in the chromosomes of the transgenic offspring. DNA integration occurs, however, at low frequencies often with only a few percent of the viable offspring identified as transgenic. This presents significant difficulties, for example, with cattle where only approximately 0.8% of the embryos used are born as transgenic animals (Wall, 1996; Pinkert & Murray, 1999). Sheep (0.88%), pigs (0.91%) and goats (0.99%) have similar efficiencies of transgenic

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animal production (Pinkert & Murray, 1999). The low frequency of transgene integration, however, is but one important limiting factor in transgenic animal production. Another key limiting factor is the embryo survival rate, or number of microinjected, transferred embryos that result in the birth of an animal (Wall et al., 1997). In both cattle and mice, typically only 15% of microinjected transferred embryos can survive to term (Wall, 1996). Overall, the use of pronuclear microinjection to generate transgenic livestock is currently a time-consuming, inefficient, and expensive process, since large numbers of embryos must be injected and transferred to obtain a transgenic animal. To make the production of transgenic livestock more practical, the inefficiencies must be overcome.

Production of transgenic animals by nuclear transfer (NT) cloning techniques has great potential, but is a developing technology that requires highly specialized techniques and is not yet consistently reliable. In addition, many NT-derived animals suffer from an array of abnormalities ranging from large offspring syndrome to respiratory and metabolic disorders (Humpherys et al., 2002). The overall frequency with which cloned animals develop to term has been estimated to be in the 2–3% range (Gurdon & Colman, 1999) and is comparable to the 1% frequency of transgenic animal production obtained with the simpler method of pronuclear microinjection. The field of applied transgenesis would benefit greatly if the efficiency of generating transgenic animals could be increased. The benefit could be greater still if it could be applied to the well-known and widely used method of pronuclear microinjection. We have been investigating the effects of modifying the state of DNA to be injected on the overall efficiency of transgenic animal production by using recombinase coated DNA for the pronuclear microinjection of fertilized zygotes.

Recombinase proteins could be useful to the field of transgenesis for several reasons. Recombinase proteins function *in vivo* to mediate DNA strand pairing and DNA strand exchange between homologous DNA segments during cellular DNA recombination and repair processes (Radding, 1982; Cox & Lehman, 1987). The recombinase A (RecA) protein from *E. coli* is one of the best characterized of the recombinases, and along with 25 cofactors, plays a central role in all homologous recombination and DNA repair events in *E. coli* (Kowalczykowski & Eggleston, 1994; Shinohara & Ogawa, 1995). RecA protein can act to bind single-stranded and double-stranded DNA, bind and hydrolyze ATP and form helical filaments (Kowalczykowski et al., 1994). RecA-like proteins also have been identified in eukaryotes, indicating a conserved function in DNA recombination and repair (Brendel et al., 1997). The eukaryotic RecA homologue, Rad51, has several properties in common with RecA including promoting homologous pairing, DNA strand exchange activity and the formation of filaments on both double and single-stranded DNA (Sung, 1994; Sung and Robberson, 1995; Sugiyama et al., 1997).

RecA binds cooperatively to single-stranded DNA to form a nucleoprotein filament with a stoichiometry of one RecA monomer for every 3–4 nucleotides of DNA (Cox & Lehman, 1987), which completely protects the DNA from digestion by phosphodiesterases and nucleases (Yu & Egelman, 1992). RecA protein-coated complementary single-stranded (css) DNA filaments (RecA-DNA) have been used to target non-denatured homologous sequences *in vitro* by the formation of stable multi-stranded DNA hybrids (Revet et al., 1993; Sena & Zarling, 1993) and thus may catalyze similar DNA hybrid recombination intermediates in pronuclear-microinjected zygotes. RecA-DNA is stable and suitable for pronuclear microinjection, and is therefore amenable for testing the effects of RecA protein-coated DNA strands on the process of transgenesis. We evaluated RecA protein-coated DNA in the production of transgenic goats and pigs as part of an experiment to generate targeted replacement of endogenous genes via pronuclear microinjection of RecA-DNA. Here, we report improved efficiency for the production of transgenic goats made by the pronuclear microinjection of RecA-DNA designed to alter the properties of milk by targeting the replacement of the endogenous goat milk β-lactoglobulin (β-lg) gene and improved efficiency of transgenic pig production made by the injection of a RecA-DNA modified α-1,3-galactosyltransferase (GalT) gene segment.

Materials and methods

Injection material – goats

All goat injection material was designed to target the goat β-lg sequences in combination with various inserted cDNAs in frame with the β-lg start codon. A 311 bp (from −157 to +154) DNA fragment of the goat β-lg gene was amplified from goat genomic DNA by polymerase chain reaction (PCR) with primers A

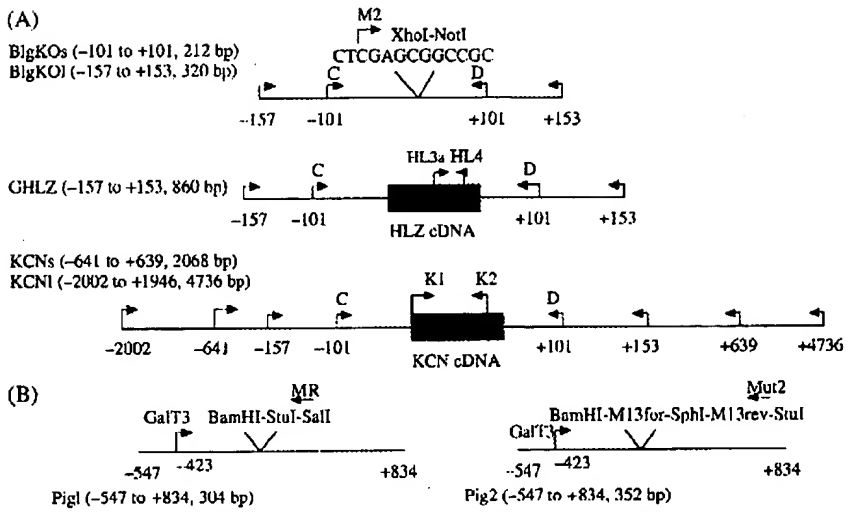

Figure 1. Schematic diagram of targeting vectors and PCR primers used for analysis. Regions of DNA used in individual vectors either side of the start codon for the β-lg gene in goats (A) and either side of the catalytic coding domain in exon 9 in the pig GalT gene (B) and inserted sequences. Arrows represent PCR primers used for the analysis of animals.

(5′-AAA TGG TAC CGG GGC CCG GGG ATG AGC CAA-3′) and B (5′-AAA TTC TAG ATG AGG CCC AGC TCC CCT GCC-3′) and cloned into pBluescript SK (Stratagene, La Jolla, CA) by the use of *Kpn*I and *Xba*I sites included in the primers. The resulting plasmid (pBLG1) was then modified by PCR to replace the translation start codon in exon 1 with a 13 bp insertion sequence (5′-GCG GCC GCT CGA G-3′) containing the unique restriction enzyme sites *Xho*I and *Not*I with primers A and F (5′-GCG GCC GCT CGA GGG CTG CAG CTG GGG TCG TG-3′) as well as B and E (5′-CTC GAG CGG CCG CAA GTG CCT CCT GCT TGC CCT-3′) for the first few cycles followed by amplification of the modified fragment with primers A and B. The resulting plasmid (pBlg-KO) contained 311 bp of goat β-lg DNA surrounding the start codon that was replaced with the 13 bp insertion sequence. The introduction of the 13 bp insertion sequence also generated a 3 bp deletion, resulting in a frame shift mutation in exon 1 of the goat β-lg gene.

A total of five goat targeting vectors were generated in a similar fashion and ranged in size from 212–4736 bp (Figure 1(A) and Table 1). All targeting vectors had the 13 bp *Xho*I, *Not*I mutation in place of the start codon. In addition, vector GHLZ had the complete 540 bp cDNA for human lysozyme (Maga et al., 1994) inserted into the *Xho*I site in the plasmid pBlgKO. The bovine kappa casein (KCN) vectors had the complete cDNA for bovine κ-casein (778 bp) inserted at the introduced *Xho*I site. The κ-casein cDNA was isolated from lactating bovine mammary tissue by standard RT-PCR methods with primers K1 (5′-CTC GAG ATG ATG AAG AGT TTT TTC CTA G-3′) and K3 (5′-CTC GAG TTT ATT ATG CAG GAA TCA A-3′). The orientations of the human lysozyme and κ-casein inserts were verified by restriction enzyme digests and DNA sequencing.

Table 1. DNA targeting vectors

Vector	β-lg or GalT regions used[a]	Inserted sequence[b]	Total length (bp)
BlgKOs	−101 to +101	13 bp mutation	212
BlgKOl	−157 to +153	13 bp mutation	320
GHLZ	−157 to +153	540 bp HLZ cDNA	860
KCNs	−641 to +639	778 bp KCN cDNA	2068
KCNl	−2002 to +1946	778 bp KCN cDNA	4736
Pig1	−547 to +834	19 bp mutation	304
Pig2	−547 to +834	48 bp mutation	352

[a] Regions of DNA used in individual targeting vectors either side of the start codon for the β-lg gene in goats and either side of the catalytic coding domain in exon 9 in the pig GalT gene.
[b] Sequence inserted in each targeting vector.

Injection material – pigs

The injection material used in pigs was designed using the catalytic coding domain of the pig GalT gene. The targeting vectors consisted of 150 bp each side of the catalytic coding domain present in exon 9 (from 547 to

834), with either a 19 bp (Pig1) or 50 bp (Pig2) DNA insertion at position 686 (Figure 1(B) and Table 1). The 19 bp insertion (5′-TAG TGG ATC CAG GCC TGT C-3′) containing the unique restriction enzyme sites *Bam*HI, *Stu*I and *Sal*I was introduced into exon 9 of the pig gene by PCR with primers GalT3 (5′-GAT AGA GCT GGG TCC TCT GCG-3′) and Mut1 (5′-CAC GAG GTG *TAG TGG ATC CAG GCC TGT C*GA CTT CCT CTT CTG CAT TGA CGT GGA TC-3′) and GalT 4 (5′-AAT GTA GGC TGC GGA CTC CTT C-3′) and Mut2 (5′-GAG GAA GAC *GTC GAC AGG CCT GGA TCC ACT A*CA CCT CGT GCT GGA TGT GGG-3′). The resulting 304 bp fragment was cloned into pBluescript and termed pPig1. Targeting vector Pig2 was generated by digesting pPig1 with *Bam*HI and *Stu*I and introducing the 48 bp oligonucleotide (5′-GGA TCC GTT TTC CCA GTC ACG ACG CAT GCC AGG AAA CAG CTA TGA CAG GCC T-3′) consisting of *Bam*HI and *Stu*I ends and a unique *Sph*I site flanked by the forward and reverse universal primers. Both targeting vectors were verified by restriction enzyme digests and DNA sequencing. All goat and pig DNA targeting vectors were used for RecA protein coating.

Conventional DNA constructs

Non-RecA protein coated construct DNA (α_{s1}-HLZ, 23.5 kb) has been previously described (Maga et al., 1994) and consists of the promoter and flanking regions of the bovine α_{s1}-casein gene with the cDNA for human lysozyme inserted in exon 1 of the casein gene. DNA construct Pig3 was an unrelated 8 kb transgene encoding a gene to model heart disease.

Preparation of injection DNA

All DNA targeting vectors and traditional DNA constructs were removed from vector sequences with appropriate restriction enzymes and purified with Elutip-D columns (Schleicher & Schuell, Keene, NH) prior to microinjection. For RecA protein coating of injection DNA, linear, double-stranded DNA (200 ng) was heat denatured at 98°C for 5 min, cooled on ice for 1 min and added to a protein coating mix containing Tris-acetate buffer, 2 mM magnesium acetate and 2.4 mM ATPγS. RecA protein (8.4 μg Roche, Indianapolis, IN) was immediately added and the reaction incubated at 37°C for 15 min then the magnesium acetate concentration was increased to a final concentration of 11 mM. The RecA protein coating of the cssDNA was monitored by agarose gel electrophoresis with uncoated double-stranded DNA as control. The electrophoretic mobility of RecA-DNA is significantly retarded as compared with non-coated double stranded DNA. RecA-DNA was diluted to a concentration of 5 ng/μl and used for the pronuclear microinjection of goat or pig zygotes. The conventional DNA constructs α_{s1}-HLZ and Pig3 DNA constructs were diluted to a final concentration of 5 ng/μl with microinjection buffer (10 mM Tris, 0.25 mM EDTA pH7.4) for microinjection.

Generation of embryos

Pronuclear stage goat embryos were obtained from donor animals in which estrus was synchronized by using progestin pessaries (Redopharm, Ltd.) for 14 days. On day 13, follicle stimulating hormone (FSH) was given twice daily (IM) over 3 days, beginning with a dose of 5 mg the first day, 4 mg the next day and 3 mg the third day if needed, with removal of the progestin sponge on day 14. Twenty-four hours after progestin removal, gonadotropin releasing hormone (GnRH Cystorelin®, Wafco Int'l.) was administered (5 mg dose, IV) to all animals, and does in estrus were bred to fertile bucks. Embryos were recovered by oviductal flushes on day 2, where day 0 is the first day of estrus. Estrus in recipient females was synchronized to correspond with the donors by use of progestin pessaries for 14 days. Microinjected embryos were surgically transferred into the oviducts of recipient does via midline laparotomy on the same day. Pregnancies were confirmed and monitored by ultrasound at days 28, 35, 47 and 54 following embryo transfer.

Pronuclear stage porcine embryos were obtained after injection of randomly selected periestrus gilts with PG600 (400 IU PMSG, 200 IU HCG, Intervet). Gilts were then grouped and sorted into new pens and feed changed from grower to sow diet. Seventy-three hours after PG600 administration, gilts were injected with 750 IU of HCG (Intervet). Gilts were fertilized with mixed semen 30 h later. Pronuclear-stage embryos were collected from oviductal flushes 19–20 h after AI. Microinjected embryos were surgically transferred to recipient animals that were synchronized by weaning, on the same day. Pregnancies were confirmed by ultrasound. All animals were housed and cared for under AAALAC-approved conditions.

Analysis of animals

Samples of umbilical cord were taken at birth, and ear notch (goats) or tail clips (pigs) were obtained within

Table 2. Targeting vectors and PCR analysis

Vector	Primer pair	Primer sequence	Product size (bp)
Goats			
Endogenous	G1/H1	G1: 5′-AGGCCTCCTATTGTCCTCGT-3′ H1: 5′-ACGTCACAGCCTCTCTTGGT-3′	369
BlgKO	C/D; M2/D	C: 5′-CCGGGCTGGCTGGCTGGCA-3′ D: 5′-TCGAACCTTCTGGATGTCCAGG-3′ M2: 5′-CAGCCCTCGAGCGGCCGC-3′	202 & 111
GHLZ	HL3a/HL4	HL3a: 5′-GAGTGGTTACAACACACG-3′ HL4: 5′-GCACAAGCTACAGCATCAGCG-3′	183
KCN	K1/K2	K1:5′-CTCGAGATGATGAAGAGTTTTTTCCTAG-3′ K2: 5′-CTCGAGTTAGACCGCGGTTGAAGTAA-3′	585
	K1a/K5r	K1a: 5′-CCCAAGAGCAAAACCAAGAA-3′ K5r: 5′-TAGGCTCACCACTAGCAATGG-3′	373
Pigs			
Endogenous	GalT1/GalT2	GalT1: 5′-GAGCATTACTTGGAGGAGTTC-3′ GalT2: 5′-GCCTATATGATAATCCCAGCAG-3′	590
Pig1	GalT3/MR	GalT3: 5′-GATAGAGCTGGGTCCTCTGCG-3′ MR: 5′-GACAGGCCTGGATCCACTA-3′	164
Pig2	GalT3/Mut2	GalT3: 5′-GATAGAGCTGGGTCCTCTGCG-3′ Mut2: 5′-GAGGAAGACGTCGACAGGCCTG GATCCACTACACCTCGTGCTGGATGTGGG-3′	164

one week of age from all offspring. DNA was prepared from tissue samples by incubation in digestion buffer (0.05 M Tris, 0.1 M EDTA, 10% SDS, 20 mg/ml Proteinase K) at 55°C overnight followed by phenol chloroform extraction. Transgenic animals were initially identified by PCR analysis. A PCR was first performed with an endogenous set of primers to serve as an internal PCR control (Table 2). For goats, the internal control amplified an endogenous 369 bp region spanning the start codon in exon 1 of the goat β-lg gene. Similarly, in pigs the internal control amplified a 590 bp fragment in exon 9 of the porcine GalT gene (Table 2).

To identify transgenic animals, PCR was performed in triplicate on each tissue sample with primer sets within each injected DNA fragment (Figure 1 and Table 2). For the BlgKO animals, a nested PCR was done first with primers flanking the mutation insertion site (C/D) followed by amplification with a mutation-specific primer (M2/D). Primers C/D generated a 202 bp fragment for all animals and a 111 bp product only if the animal was transgenic. For vector GHLZ and DNA construct α_{s1}HLZ, a 243 bp product specific to the human lysozyme cDNA was amplified with primers HL3a/HL4 if the animal was transgenic. These primers spanned exons in the human lysozyme cDNA. For KCN vectors, a 585 bp product specific to the bovine κ-casein gene was amplified with primers K1 in exon 1 and K2 in exon 4. For the Pig1 and Pig2 targeting vectors, transgenic samples were identified by PCR with primers GalT3 and MR or Mut2, respectively, primers specific for the introduced mutation (164 bp). For all PCR, a total of 0.1 μg of genomic DNA from potential transgenic offspring was added to a standard PCR reaction containing buffer, 2.0 mM $MgCl_2$, 10 mM dNTP's, 10 pmol each of primer and 2.5 U of Taq DNA polymerase in a final volume of 50 μl. Samples were subjected to a single denaturation step of 97°C for 2 min followed by 30–35 cycles of 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. For KCN animals, the annealing temperature was 55°C, for GHLZ and α_{s1}-HLZ the $MgCl_2$ concentration was 1.5 mM and for BlgKO, extension time was 30 s. Products were analyzed by standard ethidium bromide agarose gel electrophoresis. PCR products from all animals scoring positive for integrated DNA were sequenced to verify the identity of the PCR product. Multiple PCR primer sets were run for each line of animals (data not shown).

Southern blots were performed on all PCR-positive animals as well as negative control animals to verify further the presence of the injected DNA. Briefly, 15 μg of genomic DNA was digested with *TaqI* overnight and run on a 1% gel overnight at 35 V and transferred to a nylon membrane (Hybond+, Amersham Pharmacia) in 0.4 N NaOH. Membranes were probed with the appropriate probe (human lysozyme cDNA for GHLZ and α_{s1}HLZ; bovine κ-casein cDNA for KCN; injection material for BlgKO, Pig1 and Pig2) labeled by random priming with α^{32}P-dCTP. Hybridization and standard washes (0.1% SSC/0.1% SDS as final wash) were carried out at 65°C.

Results

RecA-protein coating of DNA

A total of 11 zygote microinjection experiments were carried out, eight in goats and three in pigs, to evaluate the efficiency of transgenic livestock production using DNA coated with RecA protein (RecA-DNA). Five different DNA targeting vectors were designed and generated for use in goats and consisted of either a 13 bp insertion sequence (BlgKO), or the cDNAs for human lysozyme (GHLZ) or bovine κ-casein (KCN) flanked by varying amounts of goat β-lg DNA (Figure 1(A) and Table 1). A control cDNA expression vector (α_{s1}-HLZ) containing the human lysozyme cDNA flanked by the bovine α_{s1}-casein promoter (Maga et al., 1994) was also injected into zygotes in both its conventional linear, double-stranded form and in the RecA-DNA form. Two different targeting vectors (Pig1 and Pig2) were designed using the porcine GalT gene (Figure 1(B) and Table 1) and used in the pig zygote microinjection experiments in the RecA-DNA state. The conventional expression vector Pig3 was unrelated to the pig targeting vectors and was microinjected into pig zygotes as conventional linear double-stranded DNA.

All construct DNA was successfully coated *in vitro* by RecA protein, as visualized by agarose gel electrophoresis (Figure 2), and used for the standard pronuclear microinjection of goat or porcine zygotes. RecA protein coating of cssDNA resulted in an expected shift of DNA mobility to a higher molecular weight product. This can be clearly seen in Figure 2(A) for the RecA-DNA 2 kb goat KCN vector migrating at approximately 2.5 kb and in Figure 2(B) for the RecA-DNA 300 bp Pig1 probe migrating at greater than

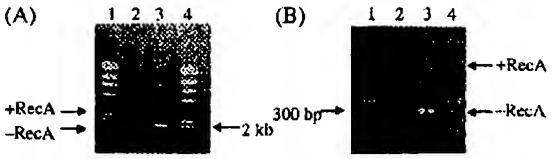

Figure 2. RecA protein coating of goat and pig DNA. (A) RecA protein coating of 2068 bp goat vector KCNs. Lane 2: 100 ng of KCNs coated with RecA protein. Lane 3: 100 ng of double-stranded KCNs DNA (2068 bp). Lanes 1 and 4: λ *Hin*dIII DNA marker. (B) RecA protein coating of 304 bp pig vector Pig1. Lane 2: 100 ng of Pig1 coated with RecA protein. Lane 3: 100 ng of double-stranded Pig1 DNA (304 bp). Lanes 1 and 4: 100 bp DNA marker. The electrophoretic mobility of the RecA-DNA is slowed compared to non-coated, double-stranded DNA.

1.5 kb. All DNA constructs used in this study ranging in size from 200 to 23,000 bp were successfully coated with RecA protein (data not shown).

Statistical analysis

The results of the zygote microinjection experiments are presented in Table 3. A pairwise comparison (Chi-square) demonstrated no significant difference between individual experiments (Table 3) and therefore, results were combined for statistical analysis. For example, the results from microinjections of goat zygotes with the RecA-DNA vectors BlgKO, GHLZ or KCN were not significantly different from each other, nor were the results of microinjections of porcine zygotes with RecA-DNA Pig1 and Pig2 vectors. Therefore, the RecA data from each species were pooled to conduct comparisons of the injection of RecA-DNA to DNA injected without RecA protein. Likewise, there was no significant difference between the two experiments when the conventional α_{s1}-HLZ DNA construct was microinjected without RecA protein coating.

Pregnancy rates

The pregnancy rates for goats receiving zygotes microinjected with RecA-DNA were consistent across all experiments with a mean of 71% ($n = 86$, range of 67–100%) of embryo transfer recipients becoming pregnant and was not significantly different from the 51% ($n = 12$, range 40–62%) pregnancy rate of goats receiving zygotes microinjected with a conventional linear, double-stranded DNA construct not coated with RecA protein (Table 3). The pregnancy rates for pigs receiving zygotes microinjected with RecA-DNA averaged 41% ($n = 12$, range 38–44%), and was not significantly different from the 29% ($n = 4$) pregnancy rate of pigs receiving zygotes microinjected with a

Table 3. Summary of RecA transgenic livestock production

Livestock	Injected DNA	RecA Protein	# Embryos transferred	# Recipient animals	# Pregnant animals (%)[a]	Embryo survival (%)[b]	# Transgenic animals (%)[c]	Transgene integration (%)[d]
Goats	BlgKOs	+	81	23	16 (69)	26 (32)	6 (23)	7.4
	BlgKOl	+	12	4	4 (100)	4 (33)	3 (75)	25
	GHLZ	+	183	42	31 (71)	58[h] (32)	20 (34)	10.9
	KCNs	+	97	21	14 (67)	26 (27)	9 (35)	9.3
	KCNl	+	142	31	21 (67)	34 (24)	6 (18)	4.2
	Pooled RecA-DNA	+				30%[j]		11.4%[k]
	α_{s1}-HLZ[e]	+	47	9	6 (67)	14 (30)	5 (36)	10.6[l]
	α_{s1}-HLZ[f]	−	88	17	7 (40)	15[i] (17)	1 (7)	1.1
	α_{s1}-HLZ[g]	−	42	8	5 (62)	8 (19)	1 (12)	2.4
	Pooled non-coated	−				18%[j]		1.8%[k,l]
Pigs	Pig1	+	378	16	7 (44)	46 (12)	14 (30)	3.7
	Pig2	+	423	13	5 (38)	30 (7)	7 (23)	2.8
	Pooled RecA-DNA	+				9.5%		3.25%[m]
	Pig3[c]	−	358	14	4 (29)	23 (6)	1 (4)	0.3[m]

[a] Percentage of pregnant animals per number of recipients.
[b] Percentage of offspring born per number of embryos transferred.
[c] Percentage of transgenic animals per live births.
[d] Percent of transgenic animals per number of embryos transferred.
[e] Conventional non-denatured double-stranded DNA construct.
[f] Year 1 injections.
[g] Year 2 injections.
[h] Three animals were born dead.
[i] One animal was born dead.
Values with the same superscripts are significantly different ([j,k,m] $P < 0.01$, [l] $P < 0.05$).

conventional double-stranded linear DNA construct without RecA (Table 3).

Embryo survival

In goats, embryo survival, or the number of microinjected transferred embryos that resulted in the birth of an animal, was significantly greater ($P < 0.01$) when RecA-DNA was used (Table 3). A mean of 30% ($n = 148$) of the microinjected, transferred embryos resulted in the birth of a kid compared with 18% ($n = 23$) embryo survival when a conventional non-coated DNA construct was used. In pigs, the same trend was seen as more animals were born when RecA-DNA was used (9.5%, $n = 76$), although this was not statistically significantly different from the embryo survival rate obtained when a conventional DNA construct was injected (6%, $n = 23$, $0.1 < P > 0.05$; Table 3).

Transgene integration

Animals were determined to be transgenic by PCR analysis of several tissues including umbilical cord, ear punch or tail samples. All DNA isolated from samples was first screened with an endogenous set of primers as control for PCR amplification (Table 2). The appropriate PCR product was observed for all samples analyzed (data not shown). A construct-specific PCR with primers located in unique segments of the injection material (Figure 1 and Table 2) was then performed on umbilical and ear (goats) or tail (pigs) DNA from all animals. Only transgenic animals that had incorporated the injected DNA at any site in the chromosomal DNA should generate a diagnostic

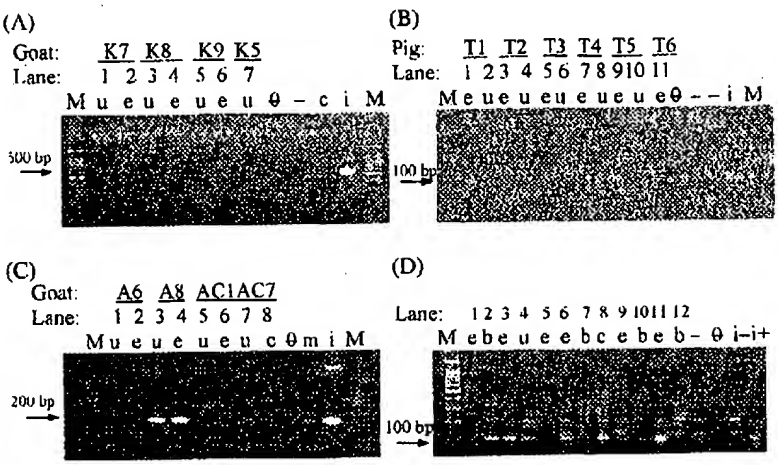

Figure 3. PCR identification of transgenic animals. Agarose gel electrophoresis of PCR products from various RecA-DNA vectors and conventional DNA constructs. Lanes designated with M are 100 bp molecular weight ladder, θ is a no DNA PCR control, – is the DNA from the uterus of a known negative embryo donor goat (A, C, and D) or pig (B), i is microinjection material, m is DNA from a human lysozyme transgenic mouse, + is DNA from a known positive transgenic goat made without RecA protein, c is genomic bovine DNA and u and e are the umbilical and ear tissue respectively of individual animals. (A) PCR identification of transgenic founder goats from the microinjection of RecA-DNA vector KCN (primers K1/K2; product size 585 bp). (B) PCR identification of transgenic founder pigs generated from the microinjection of RecA-DNA vector Pig2 (primers GalT3/Mut2; product size 164 bp). (C) PCR identification of transgenic goats from the microinjection of linear, double-stranded DNA construct α_{s1}HLZ without RecA protein (goats A6 and A8 and the microinjection of the same DNA construct in the RecA-DNA form (goats AC1 and AC7) (primers HL3a/HL4; product size 183 bp). (D) PCR identification of transgenic founders from the microinjection of RecA-vector BlgKO (primers M2/D; product size 111 bp).

PCR product. Each sample was run in triplicate, and DNA sequencing of PCR products from transgenic animals confirmed the origin of the PCR product as DNA construct-specific sequence. Examples of PCR results from transgenic and non-transgenic animals are shown in Figure 3. Diagnostic 585 bp PCR products identify two goats transgenic for the KCN DNA vector (Figure 3(A), lanes 2 and 7), while 183 bp PCR products identify transgenic goats generated from zygotes microinjected with the uncoated conventional DNA construct α_{s1}-HLZ (Figure 3(C), animal A8) and the same DNA construct injected as RecA-DNA (Figure 3(C), lane 7). Diagnostic 164 bp PCR products identify two pigs transgenic for the Pig2 DNA RecA-DNA vector (Figure 3(B), lanes 1 and 9) while a 111 bp PCR product identifies transgenic animals generated with BlgKO RecA-DNA (Figure 2(D), lanes 2–4, 6–8 and 11).

The PCR data demonstrated that the transgene integration frequency, as defined by the number of animals that was transgenic per embryo microinjected, was affected by the use of RecA. A larger ($P < 0.01$) number of founder animals were transgenic when RecA-DNA was microinjected into zygotes (Table 3). In goats, the transgene integration rate averaged 11.4% (range 4.2–25%) when RecA was used compared with 1.8% when a conventional double-stranded DNA construct without RecA protein was microinjected (Table 3). Similarly, in pigs the transgene integration rate of 3.25% (range 2.8–3.7%) was higher ($P < 0.01$) with RecA-DNA than with non-coated DNA (0.3%; Table 3). The same results were seen when a conventional DNA construct was microinjected in both the RecA-DNA and not coated forms (Table 3). An apparently higher but not significantly so, percentage of animals were born (30% coated, 18% non-coated) when RecA was used, and significantly more ($P < 0.05$) of the animals (10.6% coated, 1.8% non-coated) were transgenic.

Mosaicism

The PCR results indicated that many of the founder animals generated with RecA were mosaic (Figure 3(A)). The RecA-generated animals usually gave less consistent and weaker signals, indicative of being mosaics, even within tissue type. For instance, only the ear sample from animal K7 is positive for the transgene while the umbilical sample from the same animal is not (Figure 3(A)). The intensity of the PCR signal from transgenic animals generated with RecA-DNA was consistently weaker than those generated

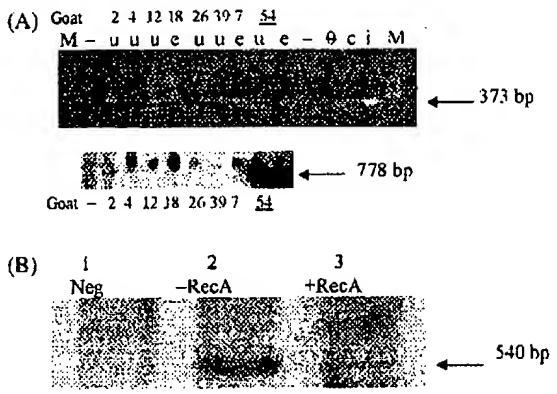

Figure 4. Southern blot analysis of transgenic animals generated with and without RecA-DNA. (A) Genomic DNA was digested with *TaqI* and probed with a α^{32}P-dCTP labeled cDNA for bovine κ-casein. RecA-DNA transgenic animals should have either a 778 or 879 bp fragment corresponding to the bovine κ-casein cDNA released from targeting vectors KCNs and KCNl, respectively (bottom panel). The top panel is a PCR reaction from the same set of animals using the primers K1a/K5r. (B) Genomic DNA was digested with *TaqI*, to release the human lysozyme cDNA (540 bp) from DNA targeting probe GHLZ and conventional DNA construct α_{s1}-HLZ, and probed with a α^{32}P-dCTP labeled cDNA for human lysozyme. Transgenic animals should have a 540 bp fragment corresponding to the human lysozyme cDNA. Lane 1: DNA from a non-transgenic animal. Lane 2: DNA from a α_{s1} HLZ transgenic goat produced by microinjection of conventional linear double stranded DNA. Lane 3: DNA from a GHLZ transgenic goat produced by the microinjection of RecA-DNA.

without RecA protein (compare Figure 3(C) lanes 3 and 4 to lane 7). The distribution of the transgene signal was more concentrated in the umbilical cord of the animals (data not shown). The mosaicism indicated by the PCR results was further confirmed by Southern blotting (Figure 4). As with PCR, signal intensity varied between different tissues of the same animal (Figure 4(A), goat 54 umbilical and ear samples). Clear Southern blots were more difficult to obtain for RecA-generated animals compared with those generated with a conventional DNA construct (Figure 4(B)). Image analysis (Image Pro Plus software) indicated that RecA-generated animals had approximately half (52%) of the number of copies as those animals generated with non-coated DNA (Figure 4(B)). Similar results were obtained for other RecA-DNA founder animals (data not shown).

Transgene transmission

Several founder animals did transmit their transgene to F1 offspring. However, transmission rates from RecA-DNA generated goats occurred at levels lower than expected, supporting the production of mosaic founders. To date, a total of 10 founder animals have been bred to non-transgenic goats to produce 55 offspring, 10 of which were transgenic by PCR. Six of these 10 founders have transmitted the transgene to at least one offspring, and each founder has produced at least two, but no more than 16, offspring.

Discussion

We investigated the possibility of exploiting the endogenous properties of recombinase proteins that allow them to associate with, bind and recombine DNA and compatibility with the common technique of pronuclear microinjection in order to generate transgenic livestock with greater efficiency and eventually with targeted sequence changes. Conditions have been determined that enable purified DNA to be stably coated with recombinase protein (Zarling & Sena, 1994; Zarling et al., 1997). The resulting RecA-DNA nucleoprotein filaments can be introduced into one-cell embryos by pronuclear microinjection and tested for biological activity. The use of RecA-DNA in the pronuclear microinjection of both goat and pig embryos resulted in significant increases in both the embryo survival rates and transgene integration frequencies. The standard pronuclear microinjection of embryos from both laboratory and livestock species results in a mean of 15% of the microinjected transferred embryos surviving to term (Wall, 1996). In these studies when RecA-DNA was microinjected, we observed an embryo survival rate that was double the expected value, with a mean of 30% of the transferred embryos in goats resulting in the birth of a kid. The embryo survival rate was as expected in goats (16%) with the use of a DNA construct without RecA protein (Ebert & Schindler, 1993). The embryo survival rates observed in pigs were lower than the expected 15% when a conventional DNA construct without RecA protein was injected (6%) but higher when RecA-DNA was used (9.5%). The overall lower numbers observed with the pigs may be attributed to the fact that fewer numbers of experiments were performed. The increased embryo survival rates indicate that RecA protein may play a role in protecting the embryo, for example, from lysis after microinjection. In cells in culture, it has been demonstrated that nuclear injection of linear plasmid DNA can induce G_1 arrest, while the injection of negatively supercoiled plasmid DNA did not induce cell cycle arrest (Huang et al., 1996). It was concluded that the presence of injected linear DNA could mimic

double-stranded breaks and thus cause cell cycle arrest via a p53-dependent pathway. A p53 pathway acts in all cells to prevent DNA damage detected in G_1 from being replicated in S phase by stopping the cell cycle. The increased embryo survival rates seen here when RecA-DNA was microinjected into embryos may be attributed to the RecA protein-mediated masking of the injected DNA and its ends from appearing to the embryo as a DNA chromosomal break. If cellular DNA damage repair pathways do not recognize the injected DNA as chromosomal damage, due to the presence of RecA protection, then cell cycle arrest will not be induced and the RecA-DNA injected embryo may have enhanced survival. It is likely that the use of DNA binding proteins from eukaryotes, such as Rad51, may have a similar impact.

The method of preparation of the DNA to be injected has been shown to influence embryo survival, but not transgene integration (Wall et al., 2000). The DNA concentration of the microinjected DNA has also been shown to affect rates of transgene integration in pigs (Nottle et al., 2001). DNA prepared with a sodium chloride gradient resulted in 22% of microinjected sheep and mice embryos surviving to term, compared to a 12% embryo survival rate with the use of a gel extraction method (Wall et al., 2000). In the work reported here, the purification method of injection material was not a factor as the same method was used for all experiments. With a gel extraction method similar to Wall et al. (2000), we obtained comparable embryo survival rates (19%) when a conventional double-stranded DNA construct without RecA protein coating was injected and yet higher embryo survival rates (30%) when RecA-DNA was used. The transgene integration rate in pigs was found to increase from 4 to 26% of animals born as transgenic with increasing concentrations of injected DNA, with concentrations between 5 and 10 ng/μl being optimal (Nottle et al., 2001). The DNA concentration used in all our microinjection experiments was a constant 5 ng/μl and was not a confounding factor. It should be noted that the transgene integration rates in pigs following the microinjection of RecA-DNA were similar to those reported by Nottle et al. (2001) at an average of 27% of live-born animals being transgenic and a much lower rate (4%) when a traditional DNA construct without RecA protein was used. This again could be due to our small sample size with pigs.

The sizes of the RecA-DNA targeting vectors used in these experiments ranged from 212–4736 bp and were, in general, smaller than most conventional DNA constructs. However, reports using DNA constructs ranging in size from 2.3 to 26 kb in livestock (Ebert & Schindler, 1993) and various sizes in mice (Brinster et al., 1985) have indicated that the length of the injected DNA does not significantly affect transgenesis rates. Due to the expense of working with livestock, it was not possible to compare directly the effect of the presence or absence of RecA protein on all individual DNA constructs. However, in the one direct comparison that was possible, although the numbers of animals were small, RecA-DNA did have an apparent effect on the integration of a large conventional DNA construct. RecA protein coating of the 23 kb α_{s1}HLZ DNA construct resulted in five transgenic founders, whereas only one transgenic founder was generated when the same DNA construct was injected without RecA protein. Although the majority of transgenic experiments use DNA constructs larger than many reported here, similar results to those with the 23 kb construct were seen with a more standard 5 kb DNA construct (data not shown) indicating that this method may be useful for more standard size DNA constructs as well.

Other methods have been investigated in an attempt to increase transgene integration frequencies. The use of repetitive sequences of bovine satellite DNA generally resulted in a higher frequency of transgene integration but also decreased embryo survival (Rieth et al., 1999). The use of short interspersed elements (SINE) in mice resulted in a four-fold increase in the integration frequency of a reporter gene (Kang et al., 2000). Three types of repetitive sequences, matrix attachment regions (MAR), SINE and microsatellites, were compared in one study, and none was found to increase significantly the integration frequency compared with controls (Pintado & Gutierrez-Adan, 2001). In all cases, experiments were carried out in embryos only, and no live offspring were produced. Restriction enzyme-mediated integration of transgenes has been demonstrated in one study in mice by co-injection of the restriction enzyme *Eco*RI along with the DNA construct. This approach was reported to double the transgene integration frequency (Seo et al., 2000). The use of RecA-DNA reported here not only enhanced embryo survival but also resulted in an overall increase in transgene integration frequency compared with previously published reports on the use of repetitive DNA sequences or restriction enzymes.

Transgene integration frequency has been found to be one of the main differences in the discrepancy in the efficiencies of production of transgenic founders

between laboratory animals and livestock. The proportion of animals that are born transgenic is generally much lower for livestock (Brem, 1985; Hammer et al., 1985). Here we show the transgene integration frequency was significantly increased in both goats and pigs when RecA-DNA was used. We observed six-fold more transgenic goats and 10-fold more transgenic pigs produced compared with the microinjection of a conventional DNA construct without RecA protein. This represents an 11-fold increase (11.4 v.s. 0.99) in goats and a three-fold increase (3.25 v.s. 0.9) in pigs in the efficiencies of transgene integration over previously published reports using standard pronuclear microinjection to generate transgenic livestock (Pinkert & Murray, 1999). In a direct comparison with goats, one study reported 1.2–2.3% (mean = 1.7%) of transferred, microinjected embryos born as transgenic kids (Ebert & Schindler, 1993) compared with 11% transgenic kids produced by the microinjection of RecA-DNA. Once bound by RecA protein, DNA has been shown to be protected from degradation by phosphodiesterases and nucleases (Yu & Egelman, 1992). The RecA protein may also act in our studies by protecting the injected DNA from degradation by cellular enzymes, thereby increasing the half-life of the DNA probes resulting in increased associations with the genome for a greater chance of producing a transgenic animal. The protection offered by RecA may also increase the probability that the transgene integrates after the one-cell stage.

Indeed, data collected by PCR analysis indicated that most founder animals were mosaic, even within tissue type, indicating that the recombinase reaction may be related to cell division and occur after the one-cell stage, thereby resulting in mosaic animals. The mosaic nature of these founder animals has made it difficult to determine if any of the integration events occurred at the targeted locus. Results have been inconsistent as often PCR data indicated that homologous recombination had taken place but Southern blots did not, or vice versa. The mosaic nature and low copy number of the founder animals can be seen in Southern blots where signal distribution and intensity are not consistent (Figure 4(A)). The generation of F1 animals should help clarify this situation. In general, when using pronuclear microinjection, 70% of founders are not mosaic and transmit their transgene to offspring (Wall, 2001). The observation that many of our founder animals are mosaic is supported by the generally low intensities and differential tissue distribution of signals consistently observed in our assays. The traditional approach to determine the level of mosaicism in an animal is by breeding and quantifying transmission of the transgene. The goats described here are currently being bred to address this issue and to determine if any is germ-line transgenic. To date, 60% of our transgenic founders have transmitted their transgene to F1 offspring. This rate of mosaicism is in the same range as that typically seen by the use of pronuclear microinjection. However, only 18% of the F1 offspring were transgenic. Additional offspring from each founder are being produced to verify these numbers. Based on the assumptions that 70% of founder animals will transmit their transgene to 50% of their F1s with the traditional microinjection of double-stranded DNA, one could expect to produce seven F1s from 20 founders from a single breeding that produced one offspring. With the use of RecA-DNA, we have seen that, on average, 10-fold more founders can be generated and that 60% of them will transmit their transgene to 18% of their F1s. Therefore, 200 founders could be generated with RecA-DNA using the same number of embryos required to produce 20 founders without RecA, and 22 F1 (three-fold more) offspring would result.

The efficiency with which transgenic animals are generated by both pronuclear microinjection and NT-based cloning is quite low. The availability of a strategy that could be applied to the simple method of pronuclear microinjection to increase the efficiency of transgenic animal production, particularly in the more commercially important livestock species, would be of significant benefit. We observed that the use of RecA-DNA increased embryo survival and enhanced the ability of the microinjected fragment to integrate into the host genome. However, many of the founder animals were mosaic and transmitted the transgene to their offspring at low frequencies. In theory, based on the data presented here, this low transmission rate can be overcome by the increases gained in embryo survival and transgene integration. On average, 10-fold more founder animals can be generated with RecA-DNA. If the level of mosaicism can be consistently overcome, then the increases in embryo survival and transgene integration frequency could lead to a decrease in the numbers of embryos, and therefore animals, required to produce a transgenic founder. This would be very desirable, especially with livestock, as it would decrease the time, expense and number of animals required to generate a transgenic line. Furthermore, the use of RecA-DNA can be easily applied to

existing techniques and is applicable to virtually any animal species.

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